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(71) Applicant *(for all designated States except US)*: **LABORATORI DERIVATI ORGANICI S.p.A.** [IT/IT]; Via M. Barozzi 4, I-20122 Milano (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DE AMBROSI, Luigi** [IT/IT]; Via G. Carducci 8, I-13048 Santhià/Vc (IT). **IANNACONE, Nicola** [IT/IT]; Via Eugenio Chiesa, 2, I-56123 Pisa (IT). **GONELLA, Sergio** [IT/IT]; Via Dolomiti, 1, I-13048 Santhià/Vc (IT). **VISMARA, Elena** [IT/IT]; Via G. Colombo, 81A, I-20133 Milano (IT). **NESTI, Solitario** [IT/IT]; Via Giugnano, 42, I-51030 S. Baronto/PT (IT). **TORRI, Giangiacomo** [IT/IT]; Via G. Colombo, 81A, I-20133 Milano (IT).

(74) Agent: **SERRAVALLE, Marco**; Serravalle s.a.s., Via B. Cellini, 11, I-20090 Segrate (IT).

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(54) Title: PROCESS FOR THE PHYSICAL DEPOLYMERIZATION OF GLYCOSAMINOGLYCANES AND PRODUCTS OBTAINED THEREFROM

(57) Abstract: The invention relates to a process for the depolymerization of glycosaminoglycanes characterized by the use of electron beam radiation, optionally in the presence of an organic compound selected from the group consisting of ethers, alcohols, aldehydes, amides and formic acid. The invention also relates to the intermediate depolymerized heparin obtained by the process. The intermediate depolymerized heparin can be dissolved in a buffer solution and fractionated by Gel Permeation for obtaining the desired Molecular Weight.

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Process for the physical depolymerization of glycosaminoglycanes and products obtained therefrom.

State of the art

Glycosaminoglycanes are natural products of large pharmaceutical interest. Among the most widely used we can mention heparin, dermatan, heparansulphate and chondroitines.

The molecular weight of the natural products varies considerably and normally ranges from 5 to 40 kDa. It is however known that for certain applications lower molecular weights lead to higher pharmacological activity. The high molecular weight of these compounds often renders impossible their oral administration. Furthermore, it is known that specific activities of glycosaminoglycanes are related to relatively short saccharide sequences. Thus, it would be very advantageous to depolymerize glycosaminoglycanes reducing the molecular weight without loosing the active sites present in the molecule.

The chemical depolymerization of glycosaminoglycanes is well known in the art and leads to glycosaminoglycanes of lower MW but also with a lower S content.

EP 394 971 discloses an enzymatic or chemical depolymerization process. The obtained heparin oligomers have a sulphur content lower than 9%.

WO 90/04607 discloses a depolymerization of heparin and dermatansulfate by the use of H_2O_2 and Cu^{2+}. The ratio SO_3^-/COO^- is slightly lower than in the starting heparin.

US 4,987,222 discloses a method for the depolymerization of heparin by the use of gamma rays. The examples disclose the preparation of heparin of Mw around 5,000 Da and with a high S content.

Summary of the invention

The present invention relates to a physical process for the depolymerization of glycosaminoglycanes by the use of electron-beam radiation (EB). It also relates to the glycosaminoglycanes obtained by this process.

Detailed description of the invention

The present invention provides a physical depolymerization process which reduces the molecular weight of glycosaminoglycanes without substantially modifying the chemical

structure of the same.

The objective is achieved through use of electron-beam radiation. When using heparin as a starting material, this process results in a low to ultra-low molecular weight heparin characterized by high S content.

The starting materials to be used in the process according to the present invention are natural glycosaminoglycanes such as heparin, heparansulphate, dermatane and chondroitine. Other suitable starting materials are derivatives of glycosaminoglycanes obtained by known methods. Thus, for example, the N-acetyl or N-sulphate groups of the residues of hexosamine can be transformed into amino groups through N-desulphation or N-deacetylation reactions and the sulphate groups of the uronic acids can give rise to epoxy groups through desulphation reactions.

In another embodiment, it is possible to use as a starting material for the process of the present invention a glycosaminoglycane which has already undergone a depolymerization process either chemical or enzymatic. The use of partly depolymerized glycosaminoglycanes is for example relevant in case of heparin which has undergone an acid pretreatment that has as a side effect partial depolymerization, or when depolymerizing functionalized glycosaminoglycanes. The conditions used for the introduction of functional groups are sometimes also causing reduction of the molecular weight of the polysaccharide.

Thus, not only it is possible to perform both steps by using electron-beam radiation, but it is possible to perform a first depolymerization step by using electron-beam radiation followed by a second step using chemical-enzymatic depolymerization, or to perform a first step of chemical-enzymatic depolymerization followed by electron-beam radiation depolymerization.

The process of the present invention allows reduction of the molecular weight of the glycosaminoglycane without sensible modification of the chemical structure of the polysaccharide.

The dose of radiation used in the depolymerization process depends on several factors, e.g. the type of glycosaminoglycanes, the desired final Mw, the energy of the radiation. In general, the dose of radiation will vary in the range 400-8,000 kGy, preferably 800-6,000 kGy, more preferably 1,200-5,000 kGy.

Preferably, the electron-beam radiation has an energy comprised between 100 and 1000 keV, most preferably between 100 and 500 keV.

The depolymerization process can be performed in a broad range of temperature, it is however preferred to maintain the temperature between 0 and 50°C, most preferably between 20 and 40 °C.

The depolymerization process according to the invention is preferably performed in aqueous solution, optionally in the presence of an organic compound selected from the group consisting of alcohols, ethers, aldehydes, amides and formic acid. Preferably, the organic compound is selected from compounds of formula I, II and III.

$$R_2\text{-CH-O-R} \qquad \text{(I)}$$

$$\begin{array}{l} \text{O} \\ \text{||} \\ \text{R-C-H} \end{array} \qquad \text{(II)}$$

$$\begin{array}{l} \qquad\quad \text{O} \\ \qquad\quad \text{||} \\ (\text{R-CH})_2\text{-N-C- R} \end{array} \qquad \text{(III)}$$

wherein each R is independently selected from the group consisting of H, OH, CHO, C_1-C_6 alkyl and acyl, optionally substituted by oxygen atoms; two R groups optionally join together to form a ring.

Preferred examples of alcohols are: methanol, ethanol, n-propanol, isopropanol, n-butanol, isobutanol, glycerol.

Preferred examples of ethers are: tetrahydrofurane, dioxane, diethylether, tertbutylmethylether, dioxolane.

Examples of aldehydes are formaldehyde, glyoxal, acetaldehyde or stabilized forms thereof (trioxane, glyoxal trimeric dihydrate).

Preferred examples of amides are: N,N-dimethylformamide, N,N-dimethylacetamide, N,N-diethylformamide, N-methylpyrrolidone.

The concentration of glycosaminoglycane in the solution to be submitted to radiation can vary in a broad range. Preferably it is comprised between 2 and 25% w/v, more preferably between 5 and 15%.

After irradiation, the solutions are optionally discolored either by using an oxidizing treatment or by passing them on proper resins. The solution is then generally purified by

precipitation in hydrophilic solvent. The obtained paste can be redissolved in water and lyophilized by vacuum distillation.

It is also possible to fractionate the intermediate depolymerized glycosaminoglycane by Gel Permeation Chromatography. The fractions containing the desired molecular weights are collected, concentrated by ultra filtration and lyophilized.

The process of the present invention is preferably performed by using a dynamic irradiation process.

With the term "dynamic irradiation process" it is meant a process wherein the irradiation is performed on a thin layer of liquid which is fluxing in front of the electron-beam window. In this way, the efficiency of the irradiation process is increased.

The process can be performed either in batch or in continuous mode. The apparatus is preferably formed of a reservoir from which the liquid moves to the irradiation area. The liquid is then returned to the reservoir.

The exposure of the solution to the electron stream can take place in different ways:

- in front of the window an inclined plane is placed, on which a thin layer of solution flows,
- in front of the window can be placed a system of thin pipes which allow the exposition of the solution to the electrons,
- the solution can flow directly on the window.

The optimal conditions of irradiation are determined through preliminary dosimetry.

The dosimetry has been performed considering the typical conditions of irradiation of the solution in terms of:

a) properties relating to the beam of electrons, i.e.
   - beam energy (measured in keV)
   - beam current (measured in mA);

b) properties relating to the geometry of the irradiation, i.e.
   - distance beam source-solution to process,
   - presence of possible shields or other nearby material that can be source of secondary radiation.

The dosimetry is in any case performed for a limited period of time, since the dose administered to the material is directly proportional to the time of the exposition and is determined in static conditions, while in reality the process is dynamic.

A possible embodiment is represented by Fig.1 wherein the solution is pumped by the pump P from the external tank R to the zone (I). During this transfer, the liquid is cooled down by the heat exchanger F. From (I) the solution falls by gravity following the surface (II), which is preferably porous so as to guaranty the formation of a uniform film on its surface. A pipe connects the area after film (II) with the tank R. The irradiation takes place on the film. The flow rate of the pump P determines the characteristics of the film (thickness, residence time in front of the irradiation window).

Experimental section

Characterization of the products

Molecular weight (Mw) was determined by size exclusion chromatography (European Pharmacopoeia 4th ed.: 2.2.30 e 2.2.46 for chromatographic techniques and 01/2002:0828 p. 1297 for method).

β- rays Irradiation

The solution irradiation process takes place inside an electron-beam apparatus.

The beam is generated by a hot cathode, constituted of a tungsten filament to whom a high voltage is applied.

The beam generation area is posed under vacuum. Such a vacuum is obtained by the combined action of two pumps, a mechanical one and a turbomolecular one.

The aspiration generated by these two pumps allows the achievement of ideal conditions for the free circulation of electrons which. Otherwise, would be slowed down by the air present around the cathode.

The beam reaches the region outside the chamber where it is generated passing through a very thin titanium film (thickness 10 μm). By their passage X rays are also generated. The solution to be irradiated is placed immediately outside this titanium film, at a distance conveniently as small as possible so that the beam exiting the film is not significantly attenuated and thus the use optimised without useless wastes.

The solution to be irradiated is circulated in proximity of the windows from where the beam exits and it is directly exposed to it. The circulation circuit is provided with an external pumping system. The solution is continuously circulated inside and outside the shielded area and therefore can be regularly sampled and fresh solution for processing can be added.

Example 1

1 l of 10 % sodium heparin solution , free of Heavy metal was prepared. The soution is transferred to the apparatus described in Fig. 1 and the circulation is started in mobile descending phase, over porous glass wool tissue of 1 mm thickness, with a flow rate of 10 l/h by using a peristaltic pump.

When starting the EB irradiation at 5 mA and 300 keV, the cooling system is activated, in order to maintain the temperature between 25 and 35 °C. The depolymerization id monitored by collecting samples, at fixed intervals, on which the Molecular Weight and the composition is determined. The variation in time is shown in Table 1.

The electron beam is stopped and the collected solution undergoes spray-drying to obtain the intermediate product which is fractionated by Gel Permeation.

Table 1

Minutes	> 10.000 Da	kGy	Mw
0	30 %	-	8.364
5	17 %	134	5.941
10	12 %	268	5.050
15	9 %	402	4.523
30	4 %	804	3.682
45	2 %	1.206	3.240
60	1 %	1.608	3.014

Example 2

The example was conducted under the identical conditions of example 1, but with an intensity of current of 10 mA.

At the end, the electron beam is stopped and the collected solution undergoes spray-drying to obtain the intermediate product which is fractionated by Gel Permeation

Table 2

Minutes	> 10.000	kGy	Mw
0	30 %	-	8.364
5	12 %	268	4.888
10	7 %	536	4.053
15	4 %	804	3.526
30	2 %	1.608	3.040
45	1 %	2.412	2.852
60	-	3.216	2.716

Example 3

The example was conducted under the identical conditions of example 1, but with a beam energy of 150 keV and a current of 5 mA. The results are reported in Table 3.

Table 3

Minutes	> 10.000	kGy	Mw
0	30 %	-	8.364
5	24 %	161	7163
10	21 %	322	6542
15	20 %	483	6337
30	17 %	966	5968
45	16 %	1449	6333
60	13 %	1932	5681
75	10 %	2415	5235
90	8 %	2898	4806

Example 4

The example was conducted under the identical condition of example 1, but with the addition of 0.4 %v/v of isopropanol. Table 4 reports the obtained results.

Table 4

Minutes	> 10.000 Da	kGy	Mw
0	30 %	-	8.364
5	20 %	134	6265
10	16 %	268	5653
15	12 %	402	4851
30	5 %	804	3760
45	3 %	1.206	3298
60	1 %	1.608	3018
75	1 %	2010	2855
80	-	2144	2780

Example 5

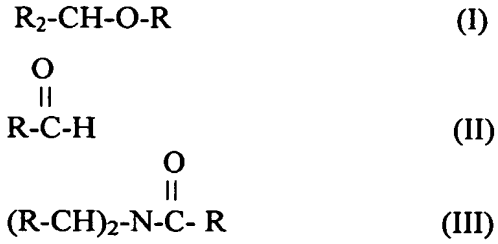
The example was conducted under the identical condition of example 2, but with the addition of 0.4 %v/v of isopropanol. Table 5 reports the obtained results.

Table 5

Minutes	> 10.000 Da	kGy	Mw
0	30 %	-	8.364
5	16 %	268	5625
10	10 %	536	4626
15	7 %	804	4043
20	4 %	1072	3559
25	3 %	1.340	3289
30	3 %	1.608	3261
45	1 %	2412	2913
55	1 %	2948	2921

Claims

1. Process for the depolymerization of glycosaminoglycanes characterized by the use of electron-beam radiation.
2. Process according to claim 1 performed by using a dynamic irradiation process.
3. Process according to claims 1-2 wherein the glycosaminoglycane is heparin.
4. Process according to claims 1-3 wherein the electron-beam radiation has an energy comprised between 100 and 1000 keV.
5. Process according to claims 1-4 wherein the process is performed in aqueous solution.
6. Process for the depolymerization of glycosaminoglycanes according to claim 5 in the presence of an organic compound represented by formulas I, II and III:



$$R_2\text{-}CH\text{-}O\text{-}R \qquad (I)$$

$$R\text{-}C(=O)\text{-}H \qquad (II)$$

$$(R\text{-}CH)_2\text{-}N\text{-}C(=O)\text{-}R \qquad (III)$$

wherein each R is independently selected from the group consisting of H, OH, CHO, C_1-C_6 alkyl and acyl, optionally substituted by oxygen atoms; two R groups optionally join together to form a ring.

7. Process according to claim 6 wherein the organic compound is selected from the group consisting of methanol, ethanol, n-propanol, isopropanol, n-butanol, isobutanol, glycerol, tetrahydrofurane, dioxane, diethylether, tertbutylmethylether, dioxolane, formaldehyde, glyoxal, acetaldehyde, N,N-dimethylformamide, N,N-dimethylacetamide, N,N-diethylformamide, N-methylpyrrolidone.
8. Process according to claims 6-7 wherein the amount of organic compound varies between 0.1 and 5%.
9. Process according to claims 1-7 wherein the amount of radiation used is comprised between 400 and 8,000 kGy.

10. Glycosaminoglycanes obtainable by the process of claims 1-9.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08B37/00 C08B37/08 C08B37/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; "Process for preparing low molecular polysaccharide and oligosaccharide thereof by photochemical or ultrasonic degradation of polysaccharides" retrieved from STN Database accession no. 136:134987 XP002255493 abstract & KR 2000 012 173 A (S. KOREA) 6 March 2000 (2000-03-06) --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mazet, J-F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; "Method for manufacturing low molecular polysaccharide and oligosaccharide thereof" retrieved from STN Database accession no. 136:185632 XP002255494 abstract & KR 2000 036 332 A (S. KOREA) 5 July 2000 (2000-07-05)	1-10
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Information on patent family members

International Application No
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